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Field Evaluation of a Native Fungus for Control of Melaleuca (*Melaleuca quinquenervia*) in Southern Florida[1]

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Abstract: A native fungus, *Botryosphaeria ribis,* was evaluated under field conditions to determine its potential to control melaleuca. Applications consisted of either wound inoculations of trees with *B. ribis* or fresh stump treatments with *B. ribis* alone or mixed with imazapyr herbicide. There was no mortality among nondefoliated trees inoculated with *B. ribis.* Mortality of *B. ribis*-inoculated trees was increased by three complete defoliation cycles. Defoliated trees inoculated with isolate BR-5 exhibited 100% mortality compared to 17% for defoliated but noninoculated trees. Wounds inoculated with *B. ribis* during winter produced longer cankers than did noninoculated wounds. Stump regrowth reduction by treatment with *B. ribis* alone was less effective than treatment with imazapyr alone. Mixtures of *B. ribis* with imazapyr or imazapyr alone at comparable concentrations did not differ in stump regrowth control.

Nomenclature: Imazapyr, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-pyridinecarboxylic acid; melaleuca, *Melaleuca quinquenervia* (Cav.) Blake #[3] MLAQU.

Additional index words: Biological control, weed control, mycoherbicide, herbicide, imazapyr, regrowth control, stump treatment, *Botryosphaeria ribis,* MLAQU.

Abbreviations: DBH, diameter at breast height; PDA, potato-dextrose agar; PDB, potato-dextrose broth; SDW, sterile deionized water.

INTRODUCTION

Melaleuca is an invasive exotic weed of environmental and economic importance to southern Florida ecosystems (Diamond et al. 1991; Hofstetter 1991). It was introduced to south Florida as early as 1900 from Australia (Meskimen 1962). Reportedly, melaleuca trees planted along Biscayne Bay originated from seeds obtained from Australia in 1906, and the eastern Everglades was aerially seeded in 1936 using seeds collected in Broward County (Turner et al. 1998). According to Turner et al. (1998), the population along the southern levee of Lake Okeechobee was established in 1941. Melaleuca invades many disturbed and undisturbed plant communities, infesting about 200,000 ha of southern Florida's natural areas (Austin 1978; Bodle et al. 1994; Hofstetter 1991). Biological characteristics such as precocity, high flowering frequency, serotiny, and fire resistance have enabled melaleuca to become an aggressive weed (Flowers 1991; Hofstetter 1991). The tree displaces native plant species, increases fire hazards, deteriorates wildlife habitat, and reduces biodiversity in invaded areas (Austin 1978; Flowers 1991; Hofstetter 1991).

Chemical, mechanical, physical, and biological methods are being tried for melaleuca control (Turner et al. 1998). Current methods combine mechanical and chemical techniques, such as girdling and spraying basal areas, and spraying freshly cut stumps with chemical herbicides (Laroche and Ferriter 1992). Integrated approaches utilizing mechanical, chemical, and biological methods (insects and pathogens) have been advocated (Bodle et al. 1994; Woodall 1982).

Attempts at using fungal pathogens as silvicides date back to the 1960s (French and Schroeder 1969; Wilson 1965, 1969). The silvicidal properties of a basidiomycetous fungus, *Chondrostereum purpureum* (Pers. *ex* Fr.) Pouzar, on various weedy shrubs and trees is currently being evaluated in the Netherlands and Canada (de Jong et al. 1990; Wall 1994). Dorworth (1995) evaluated an ascomycetous fungus *Nectria ditissima* Tul. for control of red alder (*Alnus rubra* Bong.) in Canadian forests. An exotic rust fungus, *Uromycladium tapperianum,* has

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[3] Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds,* Revised 1989. Available from WSSA, 810 East 10th Street, Lawrence, KS 66044- 8897.

been released in South Africa to control *Acacia saligna* (Labill.) H. L. Wendl., a weedy leguminous tree (Morris 1991, 1997).

Biological control strategies may supplement the current melaleuca control efforts in southern Florida. More than 450 herbivorous insects are recorded on melaleuca trees in Australia (Balciunas et al. 1994). The leaf weevil *Oxyops vitiosa* Pascoe (Coleoptera: Curculionidae) was the first biocontrol agent released on Florida's melaleuca (Wineriter and Buckingham 1997). A native canker fungus, *Botryosphaeria ribis* Gross. & Duggar, found in southern Florida proved to be pathogenic to melaleuca trees (Rayachhetry et al. 1996b). Greenhouse experiments showed this to be an aggressive pathogen of trees stressed by low temperature, drought, or defoliation (Rayachhetry et al. 1996a).

Weed-killing efficacy of some pathogens may be enhanced by integration with herbicides (Hoagland 1996). Such integration may minimize the damage to nontarget plants and the environment by reducing the amount of chemical applied (Christy et al. 1993; Hoagland 1996). In vitro studies have demonstrated that hyphal inoculum of *B. ribis* was not affected by 12 to 60 mg ai/ml of imazapyr, a chemical herbicide used to prevent the regrowth of melaleuca stumps in southern Florida (Anonymous 1994; Rayachhetry and Elliott 1997). The purpose of the research reported herein was to evaluate the mycoherbicidal efficacy of *B. ribis* under field conditions in southern Florida on both standing trees and freshly cut stumps.

MATERIALS AND METHODS

March 1994 Inoculation. Six *B. ribis* isolates, BR-1 through BR-6 (Rayachhetry et al. 1996c), were used in these studies. Hyphal inoculum of each isolate was produced and used in the following manner. Monoconidial cultures were grown on potato dextrose agar (PDA)[4,5] for 3 d at 25 C and in a 12 h light cycle. A 5-mm diam disk, removed from the colony margin of each isolate, was macerated in 5 ml sterile deionized water (SDW). Three drops of the resulting suspension were added to 200 ml of potato dextrose broth (PDB)[4] in a 500-ml Erlenmeyer flask and shaken at 100 rpm using a rotary shaker for 4 d in a 12 h light cycle. Mycelia were aseptically harvested and filtered through sterile cheese cloth. The wet mycelial mats were weighed and blended for 15 s under aseptic conditions, and a 40% mycelial suspension (wet wt/v) was prepared using SDW (Rayachhetry et al. 1996b). To facilitate tree inoculations, 10 ml of this inoculum was containerized into ARBOR™ plastic capsules.[6] The plastic containers allowed delivery of the inoculum into holes drilled into tree stems.

A melaleuca stand was selected in Palm Beach County. Trees 2 to 10 cm diam at breast height (DBH) were wounded by drilling 5 mm diam by 10 mm deep holes into the stem at breast height. Ten milliliters of inoculum was injected into the sapwood by inserting the inoculum-loaded plastic container into the predrilled holes. Twenty-five trees were inoculated for each of the six isolates (henceforth referred to as treatments). A control treatment consisted of 25 similarly wounded trees injected with deionized distilled water. The containers were left attached to the trees for 12 wk. After establishment of the experiment, some trees were removed or damaged by road maintenance crews in the following treatments: control (4 trees), BR-4 (5 trees), BR-5 (1 tree), and BR-6 (1 tree). Therefore, unequal numbers of trees were available for the following evaluations and subtreatments.

Three to five trees from each of the seven treatments were harvested 3 (June 1994), 17 (August 1995), and 35 (February 1997) mo after inoculation. Trunk segments of 40 to 75 cm length containing the inoculation point at the midsection were removed and split longitudinally through the inoculation point, and the length of the discolored sapwood was measured.

Five to nine trees from each of the seven treatments were completely defoliated during September 1995 by manually removing all twigs measuring 1 cm diam or less, stripping leaves from all stems with diameters more than 1 cm, or both. The defoliated trees were revisited in November 1995 and January 1996, and all new regrowth was removed each time. In February 1997, these trees were evaluated for mortality.

Winter Inoculation. During December 1995–January 1996, this field experiment was established in two locations (Palm Beach and Broward counties). Four *B. ribis* isolates, BR-1 through BR-4 (Rayachhetry et al. 1996c), were used in this experiment. *Botryosphaeria ribis* isolates were grown on PDA for 3 d at 25 C in a 12 h light cycle. Five 3-mm diam disks were removed from the colony margin of each isolate, added to 500 ml of PDB, incubated at room temperature with 10 h fluo-

[4] Difco Laboratories, Detroit, MI 48232.

[5] Mention of trade name, proprietary product, or specific equipment does not constitute a warranty by the University of Florida, the U.S. Department of Agriculture, or the South Florida Water Management District and does not imply their approval to the exclusion of other products that also may be suitable.

[6] Tree Technology Systems, Inc., 1014 Rein Road, Cheektowga, NY 14225.

rescent light for 2 wk, and filtered through sterile cheese cloth to obtain wet mycelia. One hundred grams of the wet mycelia containing 97.5% water was added to 100 ml of SDW and blended 3 to 4 min using a commercial blender to obtain ca 1% (10 mg mycelia/ml, dry wt/v) mycelial suspension. An equal volume of the resulting mycelial suspension of four isolates was mixed together and used as inoculum stock. For each location, a fresh batch of inoculum suspension was prepared.

Trees with a DBH range of 2 to 10 cm were wounded with the sharp edge of a metal hammer. These wounds, designed to expose sapwood, were 0.5 to 1.5 cm wide and 5.0 cm long. Each of the two treatments, wound alone or wound plus *B. ribis* inoculum, was applied to 10 trees. The *B. ribis* suspension was diluted (1:9) with deionized water, and ca. 1 mg/ml of the resulting mycelial suspension (dry wt/v) was sprayed until the wounds were thoroughly soaked. In February 1997, trees were evaluated for mortality and/or canker length (distal and proximal extension of necrosis from the inoculation point).

Stump Treatments with *B. ribis* and Imazapyr. Four *B. ribis* isolates, BR-1 through BR-4 (Rayachhetry et al. 1996c), and a commercial formulation of imazapyr (Arsenal®,[7] 28.7% ai) were used in this experiment. Production and processing of ca. 1% (dry wt/v) of the inoculum stock of *B. ribis* were the same as for those described in the "Winter Inoculation" experiment. For each location, a fresh batch of inoculum was prepared and mixed with imazapyr and/or water 1 to 2 h before stump treatment. The final concentration of inoculum in spray treatments was 3.6 to 5.2 $\times$ 10^5 colony-forming units (viable hyphal fragments)/ml.

Four locations in Broward and Dade counties of southern Florida were selected to represent three kinds of habitats. The habitats included a permanently wet location with water 0.3 to 1.3 m deep, a dry location with no standing water evident throughout 1996, and two seasonally wet locations with water 0 to 0.3 m deep (standing water for 8 mo and dry for 4 mo). Water depths were 1.2 and 0.15 m at the permanently wet and seasonally wet locations, respectively, at the beginning of the experiment in October/November 1995.

Each location received 10 treatments, and each treatment was applied to three groups of 10 trees (30 trees/treatment) randomized within each location. The 1,200 trees (300 trees/location) in four locations had a minimum, maximum, and average diameter of 0.5, 11.7, and 4.4 cm, respectively, at the cut surface. The 10 treatments included water alone, *B. ribis* alone, imazapyr alone at the concentration of 12, 24, 60, and 120 mg/ml, and each of these imazapyr concentrations supplemented with a mycelial slurry of *B. ribis*. Trees in the wet or dry location were cut 15 to 30 cm above water or ground level using a machete. Trees were cut to produce V-shaped stumps and were sprayed immediately with treatments until the surface appeared thoroughly wet (ca. 0.074 ml/cm^2). The sites were revisited at the end of 3, 6, 9, and 15 mo after treatment initiation for evaluation of regrowth (sprouting) and mortality (stump decay). A stump with one or more sprouts above ground at the time of evaluation was considered regrown.

Tissue samples, about 5 $\times$ 5 mm, were removed from an area located 10 cm distally from the point of inoculation. These samples were surface sterilized by quickly dipping them into 95% ethanol followed by momentary flaming. The flamed samples were plated on acidified PDA and incubated for 14 d at 25 C in a 10-h photoperiod. The plates were then evaluated for the presence of *B. ribis* based on the morphology of the colony, mycelia, and/or conidia.

Data Analyses. Length of sapwood discoloration and stump regrowth data were analyzed using the SAS software package (SAS 1985). Analyses of variance and means separations were performed after arcsine transformation of percentages. Chi-square test and Fisher's exact test were performed using Sigma Stat (Fox and Ulrich 1995).

RESULTS AND DISCUSSION

March 1994 Inoculations. Three months after inoculation all *B. ribis*-inoculated plants developed expanding discoloration in the sapwood, compared to the noninoculated controls (Table 1). Sapwood discoloration occurred more rapidly during the first 3 mo after inoculation than during the remaining period. Length of sapwood discoloration was greater among the *B. ribis* treatments compared to control treatments on all three evaluation dates. Isolates BR-2, -3, and -4 had similar sapwood discoloration lengths in June 1994. Isolate BR-4 caused significantly more sapwood discoloration than the other isolates at 17 and 35 mo after inoculation.

Wounds in the control and most *B. ribis*-treated trees were enclosed by callus ridges within 16 mo. Under greenhouse conditions, a majority of *B. ribis*-inoculated wounds developed open stem cankers (Rayachhetry et al. 1996b). Under natural field conditions, melaleuca

[7] American Cyanamid Co., Wayne, NJ 07470.

Table 1. Mean length of sapwood discoloration of melaleuca trees wound-inoculated with six *Botryosphaeria ribis* (BR) isolates in Palm Beach County, FL, in March 1994.

Observation date	Isolate[a]						
	Control	BR-1	BR-2	BR-3	BR-4	BR-5	BR-6
	cm						
June 1994	0.6 c	5.2 bc	7.8 ab	7.0 abc	9.2 a	4.5 c	6.2 bc
August 1995	1.8 c	8.0 b	7.0 b	7.2 b	12.6 a	6.0 b	6.7 b
February 1997	1.9 c	8.0 b	9.1 b	7.5 b	13.8 a	7.1 b	7.0 b

[a] Values are means of three to five replicate trees. Means followed by the same letter(s) in the same row are not significantly different according to Waller–Duncan *k*-ratio *t*-test at P = 0.05.

trees callus rapidly despite *B. ribis* colonization of the sapwood. However, these callus tissues are invaded by *B. ribis* through the interface between the callus ridge and the sapwood (Rayachhetry et al. 1996d).

No mortality occurred among the control or nondefoliated *B. ribis*-inoculated trees, but defoliation increased mortality (Table 2). Some mortality (17%) was observed among wounded and defoliated trees that were not inoculated with *B. ribis*. In contrast, mortality of defoliated, inoculated trees was greater and varied from 22% for isolate BR-2 to 100% for isolate BR-5. Overall chi-square distribution of live and dead trees showed significant difference (P = 0.028) between inoculated and noninoculated treatments. A pair-wise comparison of the control with each of the six BR isolates showed that only isolate BR-5 significantly contributed to tree mortality (P = 0.008). The isolates BR-1, -3, -4, and -6 contributed > 50% mortality of defoliated trees but were not statistically distinct at P ≤ 0.05. In general, examination of the cross-sectional areas of control and *B. ribis*-inoculated trees at 1 cm proximal to the inoculation point revealed a relatively higher proportion of discolored sapwood and phloem tissues in defoliated than in corresponding nondefoliated trees described previously.

The efficacy of microbes as herbicidal agents may vary between greenhouse and field conditions (Charudattan 1989). The mortality caused by *B. ribis* on field-grown melaleuca trees was enhanced by repeated defoliations. These results corroborate other host–pathogen relationships in which defoliation is reported to weaken and predispose plants to various pathogens (Old et al. 1990; Schoeneweiss 1967, 1981; Wargo et al. 1973).

Table 2. Mortality of melaleuca trees wound-inoculated with *Botryosphaeria ribis* (BR) isolates in March 1994 and completely defoliated in September and November 1995 and January 1996.

Treatments	Trees evaluated	Mortality	P[a]
	no.[b]	%	
Control	6	17	—
BR-1	6	66	0.242
BR-2	9	22	1.000
BR-3	8	50	0.301
BR-4	5	80	0.080
BR-5	6	100	0.008
BR-6	8	63	0.138

[a] P values were generated by Fisher's exact test by comparing the control with each fungal isolate (BR treatments).

[b] Overall chi-square = 14.176 (6 df, P = 0.0028) distribution for live and dead trees was significant.

Winter Inoculation. No tree mortality was observed during the experimental period (data not shown). Differences in canker length due to location differences (Palm Beach and Broward counties) were not significant (Pr > *F* = 0.2418) after 1 yr. However, the treatment (wound alone and wound plus *B. ribis*) differences for both locations were significant (Pr > *F* = 0.0001). Cumulative (data pooled together for both locations) mean canker length for the wound plus *B. ribis* treatment was 31.2 cm, whereas it was 9.2 cm for the wound alone treatment. Observations after 9 mo showed a 75 to 100% wound closure by callus development in the control treatment compared to vertically expanding cankers in the *B. ribis*-treated wounds. Rayachhetry et al. (1996a) obtained similar results when greenhouse-grown melaleuca trees inoculated with *B. ribis* were exposed to low temperature treatments (0 and 6 C) before and after inoculation. This suggests that application of *B. ribis* on melaleuca during winter may enhance canker disease development. A similar phenomenon has been reported for other host–pathogen relationships (Schoeneweiss 1974; Wene and Shoeneweiss 1980).

Stump Treatments With *B. ribis* and Imazapyr. Because the commercial formulation of imazapyr is compatible with *B. ribis* in vitro (Rayachhetry and Elliott 1997), different concentrations of imazapyr and *B. ribis* mixtures were evaluated in the field. The phloem, cambium, and sapwood of control (water only) stumps at the cut surfaces remained lightly tan-colored 1 wk after treatment, whereas the cut surfaces of stumps treated with *B. ribis* alone, imazapyr alone, or *B. ribis* plus imazapyr became black. The cut surface of stumps in the control treatments callused at the outer sapwood–cam-

Table 3. Regrowth (sprouting) of melaleuca stumps treated with water, fungus (*Botryosphaeria ribis*) alone, imazapyr alone, or fungus–imazapyr mixture.

Treatment	Imazapyr concentration	Fungus	Stumps with regrowth (mo after treatments)[a]			
			3	6	9	15
	mg/ml		%			
1	0	–	37.50 a	77.50 a	84.17 a	95.83 a
2	12	–	1.67 b	3.33 b	1.47 b	4.17 c
3	24	–	0.00 b	0.00 b	0.83 b	2.50 c
4	60	–	0.00 b	0.00 b	0.00 b	0.83 c
5	120	–	0.83 b	3.33 b	3.34 b	3.34 c
6	0	+	11.66 ab	73.33 a	82.50 a	84.17 b
7	12	+	0.00 b	1.67 b	1.67 b	0.83 c
8	24	+	0.83 b	0.00 b	0.00 b	0.00 c
9	60	+	0.00 b	0.00 b	0.00 b	0.00 c
10	120	+	0.00 b	0.00 b	0.00 b	0.00 c

[a] Values are means of 120 replicate trees. Means followed by the same letter(s) in the same column are not significantly different according to Waller–Duncan *k*-ratio *t*-test at P = 0.05.

bial region; sprouts often originated from these calluses at the surface. Thus, overall regrowth of stumps in the control treatment occurred at or near the cut surfaces. Stumps treated with *B. ribis,* imazapyr, or *B. ribis* plus imazapyr regrew primarily from the lower portion of the stumps.

Overall stump regrowth percentages for three habitats (permanently wet, seasonally wet, and dry) were not different ($Pr > F = 0.1087$). Similarly, the interaction between habitat and treatments also was not significant ($Pr > F = 0.1819$). However, the regrowth of the stumps was significantly different among treatments ($Pr > F = 0.0001$) within habitat.

Cumulative stump regrowth across all habitats at 3, 6, 9, and 15 mo after treatment is presented in Table 3. Approximately 96% of the stumps in the control treatment had regrown after 15 mo. At the same time, 84% of the stumps in the *B. ribis* alone treatment had regrown, while regrowth from stumps treated with imazapyr alone or imazapyr plus *B. ribis* was less than 5%. No regrowth occurred when 24 mg/ml or greater imazapyr was supplemented with *B. ribis. Botryosphaeria ribis* alone appeared to delay regrowth from stumps but did not provide an improvement over water-treated controls. Regrowth was initiated at or near the root-collar region of *B. ribis*-treated stumps.

Attempts to reisolate *B. ribis* from *B. ribis*– imazapyr-treated stumps that were at various stages of deterioration yielded mostly bacterial and other fungal contaminations, but *B. ribis* was isolated at low levels. *Botryosphaeria ribis* is known to be obscured on PDA plates when grown in a mixture with other saprophytes (Rayachhetry et al. 1996b).

Currently, a minimum of 60 mg/ml imazapyr is used for stump treatment of melaleuca (Anonymous 1994). In our study, 12 mg/ml imazapyr alone or mixed with the fungus reduced stump regrowth to less than 5% at the end of a 15-mo evaluation period. The effectiveness of the low herbicide concentration may be due to an increased herbicide absorbing surface provided by the V-cut used in this study rather than the normal slant-cut. Addition of *B. ribis* to imazapyr did not increase regrowth control over corresponding concentrations of imazapyr alone. The efficacy of regrowth control by lower than recommended concentrations of imazapyr alone or *B. ribis* plus imazapyr mixtures may be further enhanced by improved formulations and better delivery systems.

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